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1645

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Darji et al.

Serial No.: 09/419,545

Filed: October 18, 1999

For: ATTENUATED SALMONELLA STRAIN USED AS A VEHICLE FOR ORAL IMMUNIZATION

Group Art Unit: 1645

Examiner: Sarvamangala J.N. Devi

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May 24, 2004

James P. Zeller
Reg. No. 28,491

RESPONSE TO REQUEST FOR INFORMATION

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In response to the "Request for Information" dated March 24, 2004, submitted herewith is a certified copy of EP 97106503.2 filed April 18, 1997, the priority of which is claimed under 35 U.S.C. § 119. The application was filed in the name of Gesellschaft fuer Biotechnologische Forschung mbH (GBF), and no designation of inventor was filed with the application.

The title of the application is "Salmonella typhimurium strain". The application was published October 29, 1998, as WO 98/48026.

Respectfully submitted,

MARSHALL, GERSTEIN & BORUN LLP

By: _____
James P. Zeller
Reg. No. 28,491
Attorneys for Applicants

May 24, 2004

6300 Sears Tower
233 South Wacker Drive
Chicago, Illinois 60606-6357
(312) 474-6300

Europäisches Patentamt	European Patent Office	Office européen des brevets

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Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr.	Patent application No.	Demande de brevet n°
	97106503.2	

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk

Anmeldung Nr:
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Gesellschaft für Biotechnologische
Forschung mbH (GBF)
Mascheroder Weg 1
D-38124 Braunschweig
ALLEMAGNE

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Salmonella typhimurium strain

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PATENTANWALTE
EUROPEAN PATENT ATTORNEYS
EUROPEAN TRADEMARK ATTORNEYS

BEREITERANGER 15
D-81541 MÜNCHEN

PAe BOETERS & BAUER
BEREITERANGER 15, D-81541 MUNCHEN

DIPL.-CHEM DR HANS D BOETERS
DIPL -ING ROBERT BAUER
PHYS DR ENNO MEYER

TELEFON (089) 65 00 86
TELEFAX (089) 65 39 62

April 17, 1997/hl

Our ref.: 8579
New European Patent Application
Gesellschaft fuer Biotechnologische Forschung mbH (GBF)
Weiß et al.; *Salmonella typhimurium* strain

An attenuated strain of *Salmonella typhimurium* has been tested as vehicle for oral genetic immunization. Eukaryotic expression vectors containing the genes for β-galactosidase, or truncated forms of ActA and listeriolysin - two virulence factors of *Listeria monocytogenes* - that were controlled by an eukaryotic promoter have been used to transform the strain *S. typhimurium aroA*. Multiple or even single immunizations with these transformants induced a strong cytotoxic and helper T cell response as well as an excellent antibody response. Multiple immunizations with listeriolysin transformants protected the mice completely against a lethal challenge of *L. monocytogenes*. Partial protection was already observed with a single dose. ActA appeared not to be a protective antigen.

The strength and the kinetics of the response suggested that the heterologous antigen were expressed within the eukaryotic host cells following transfer of plasmid DNA from the bacterial carrier strain. Transfer of plasmid DNA could be unequivocally shown *in vitro* using primary peritoneal macrophages. The demonstration of RNA splice products and expression of β-galactosidase in the presence of tetracycline - an inhibitor of bacterial protein synthesis - indicated that the gene was expressed by host cells rather than bacteria. Oral genetic immunization with *Salmonella* carriers provides a highly versatile system for antigen delivery, represents a potent system to identify candidate protective antigens for vaccination, and permits efficacious generation of antibodies against virtually any DNA segment encoding an open reading frame.

Introduction

The design of efficient vaccines against infectious diseases remains a major challenge in medical science. Low cost, non-invasive administration, life-long protection by single doses combined with ease of preparation, storage and transport are desirable goals to be achieved. In this respect, live attenuated bacterial carriers that express heterologous antigens are attractive vehicles for the oral delivery of vaccines. This type of delivery should result in a broad spectrum of both mucosal and systemic immune responses. Use of vaccine vectors overcomes some of the limitation of oral delivery of proteins, which usually need to be co-administered with adjuvant proteins such as cholera toxin to evoke an immune response. In addition, administration of live replicating vectors might be advantageous over other forms of administration such as microencapsulation because of the immunomodulatory properties of cell wall components of bacteria for example. Finally the natural route of entry could prove to be of benefit since many bacteria like *Salmonella* egress from the gut lumen via M cells into Peyer's Patches and migrate eventually into lymph nodes and spleen, thus allowing targeting of vaccines to inductive sites of the immune system.

Genetic immunization has recently provided a new promising approach to the vaccination problem. Isolated plasmid DNA - introduced into muscle or skin of the host - leads to expression of antigen in the host cells due to eukaryotic control elements. This has lead to B and T cell stimulation and to protective responses. How these responses are generated remains unclear. Muscle cells apparently express low levels of MHC class I but lack MHC class II and costimulatory molecules. Although, it is not known which cells function as antigen presenting cells (APC) under these circumstances, it is likely that resident dendritic cells or macrophages capture the antigen and migrate to lymph nodes and spleen to stimulate $CD4^+$ and $CD8^+$ T cells. Indeed antigen expressing dendritic cells have been observed after genetic immunization into the skin using a gene gun. It is not known whether DNA is also transferred into dentritic cells when plasmids are applied into muscles.

Several advantages have been observed with genetic immunization over conventional vaccination. The DNA can be detected for a considerable period of time thus acting like a depot

of antigen. Sequence motifs in some plasmids are immunostimulatory and can function as adjuvant. Co-expression of cytokines enhance the response and offer the possibility of modulating the induction of an immune response into a desired direction. However, many obstacles need to be overcome before general applicability can be achieved.

If it would be possible to deliver plasmids for genetic immunization with an attenuated bacterial carrier the advantages and versatilities of both systems would be combined. In addition, the natural route of administation would deliver DNA to cell types which have specifically evolved to induce immune responses. *Salmonella spp.* for this purpose are particularly suited because of the extensive knowledge on the genetics and physiology of many strains. A large body of documentation exists on their utility as heterologous antigen carriers that are capable of inducing protective immune responses. Also, safe attenuated strains of *Salmonella* are available and are already in use as vaccines in animal husbandry and man. Finally, recombinant plasmids constructed in laboratory strains of *E. coli* can be directly introduced into *Salmonellae* without further manipulations.

Here we report that orally administered *S. typhimurium aroA* carrying plasmids encoding β-galactosidase, or truncated forms of ActA or listeriolysin under the control of an eukaryotic promoter induce an efficient humoral and cellular immune response. Indeed, the strength and kinetic of the response is only compatible with the interpretation of a transfer of the expression plasmid from the *Salmonella* carrier to the nucleus of APC of the host.β-galactosidase (β-gal) activity was detectable even five weeks after administration of the oral vaccine. In addition, *in vitro* experiments with mouse primary macrophages demonstrated an efficient transfer of plasmid DNA from attenuated bactera into the nucleus of phagocytic host cells.

Results

To achieve genetic immunization with a live attenuated bacterial carrier three plasmids were used which are based on the commercially available plasmid pCMVβ. This plasmid contains the structural gene of β-galactosidase (β-gal) under the control of the human CMV early promoter and includes a splice donor and two splice acceptor sites in between the promotor and the structural gene. For studies examining the efficiency of the immune response against pathogens the β-gal gene was replaced by genes encoding two virulence factors of *Listeria monocytogenes.* A truncated gene encoding a non-hemolytic variant of listeriolysin (pCMVhly) from amino acid positions 26 to 482 and a truncated variant of the structural gene of the membrane protein ActA (pCMVActA) encoding amino acid 31 - 613 were used. *S. typhimurium aroA* strain SL7207 was transformed with these three plasmids and groups of mice were orally immunized by feeding 10^8 organisms to each mouse per immunization. This dose was found to be optimal (data not shown). The mice did not show any overt signs of illness using this immunization schedule.

Induction of a strong T cell response by immunization with Salmonellae carrying eukaryotic expression vectors

The working hypothesis of these experiments was that orally administered *S. typhimurium aroA* would result in uptake of the bacteria by macrophages and possibly dendritic cells, with concomitant activation by the endotoxin of the bacteria. Following a few rounds of bacterial division the intracellular bacteria would die because of their inability to synthesize aromatic amino acids. During this process plasmids would be released and transferred into the cytosol and the nucleus of the infected cells. Eventually, the encoded genes will be expressed by host APC.

The first prediction of this hypothesis is the induction of a strong cytotoxic response of CD8 T cells, since antigen whould be expressed in the cytosol which is the cellular compartment responsible for MHC class I presentation. The experiments were carried out in two ways. Mice were infected orally once with recombinant *Salmonellae* and their cytotoxic T cell responses were followed for several weeks by testing their spleen cells directly *ex vivo* (data not shown)

or after one restimulation *in vitro*. Alternatively, mice were orally immunized four times with two weeks intervals in between immunizations. The course of the cytoxic response was tested. Fig. 1 demonstrates that a strong and specific CD8 T cell response can be elicited with orally administered *Salmonella* carrying eukaroytic expression plasmids. Mice immunized with the truncated gene of listeriolysin elicited only a response towards targets sensitized with the immunodominant peptide comprising AA91-99 of listeriolysin (LLO) and not against targets sensitized with soluble HEL or a control peptide (Fig. 1A). Similarly, spleen cells from mice immunized with *Salmonella* carrying the ActA expression plasmid could only respond to ActA (Fig. 1D). To reveal the cytotoxic response against ActA, we exploited the pore-forming activity of listeriolysin. This activity of listeriolysin allows the introduction of soluble passenger proteins into the cytosol of target cells. Stimulators and target cells were therefore sensitized with a mixture of soluble ActA and LLO. A specific response was observed only when the combination of ActA and LLO was used. No response was found when LLO alone was tested. These responses were of the same specificity during the whole time period indicated in Fig. 1 panels B & C and E & F and when the response of mice immunized with *Salmonella* harboring the β-gal control plasmid was studied (data not shown).

The kinetic of the responses indicated that even a single dose elicited a strong cytotoxic T cell response which peaked 5 weeks after immunization and then slowly declined (Fig. 1C and F). On the other hand, the response was still rising even at the end of the observation period, i. e. 5 weeks after the last challenge in mice that had received four immunizing doses (Fig. 1B and E). Thus, a strong cytotoxic response was observed when using *Salmonella* as potential vehicle for genetic immunization.

Genetic immunization usually also evokes a CD4 helper T cell response. Therefore, cells from spleen and mesenteric lymphnodes of the same mice used above were tested for their proliferative response against soluble proteins. This type of response is mainly due to presentation of antigen via MHC class II molecules and carried out by CD4 T cells. As shown in Fig. 2, a strong and specific helper T cell response, in parallel to the cytotoxic response is observed when eukaryotic expression plasmids carried by *Salmonellae* were used for immunization (Fig. 2A and D). As with the CD8 response, a single dose was sufficient for a

good response which was still increasing at the end of the observation period regardless of whether listeriolysin or ActA was used as antigen (Fig. 2C and F). Four immunizations, however, resulted in an even stronger response which appeared long lasting since the response apparently was still increasing five weeks after the last challenge (Fig. 2B and E). Similar results were obtained with *Salmonella* carrying the control plasmid expressing β-gal (data not shown). Analysis of the supernatants of the *in vitro* cultures revealed production of IFNγ by these T cells. No IL-4 could be found, suggesting that such an immunization scheme is mainly inducing a TH1 or inflammatory type of T helper response.

Induction of specific antibodies by immunization with Salmonellae carrying eukaryotic expression vectors

Following genetic immunization, proteins are generally expressed in the cytosol since they usually do not contain signal peptides required for secretion. Nevertheless, antibody responses are evoked. We, therefore, tested the pooled sera of the groups of mice used above for presence of specific antibodies. In addition to a cytotoxic and helper T cell response, immunization with *Salmonellae* carrying eukaryotic expression plasmids induces strong and specific antibody responses as revealed by ELISA (Fig. 3A and B) or immunoblot (data not shown). Again a single immunization was sufficient for a good response which peaked four weeks after the administration of the bacteria and then declined in a fashion similar to the cytotoxic response (Fig. 3A and B). Four immunizations did not increase the antibody titer significantly but probably resulted in a longer lasting response since a plateau of antibody titer was not reached at the end of the observation period (Fig. 3A and B).

The analysis of the subclass distribution of individual mice at week 11 revealed a high concentration of IgG2a while the concentration of IgG2b and IgG3 are negligible (Fig. 3 C and D). This is in agreement with the finding that only IFNγ and no IL-4 could be detected in the supernatant of the restimulated T helper cells. However, also IgG1 was observed at high concentration in the immune sera. This subclass is found when TH2 helper cells are taking part in the immune response, suggesting that under our experimental conditions TH2 cells might also

be induced but are not revealed in the *in vitro* T cell assay.

Taken together the results presented in Fig. 1-3 show that immunization with *S. typhimurium aroA* carrying eukaryotic expression vectors can evoke responses in all three specific effector compartments of the immune system, namely, cytotoxic CD8 T cell, CD4 T cells and antibodies. The T helper compartment is strongly biased towards a TH1 or inflammatory T helper response.

Protection against lethal doses of L. monocytogenes

The strong response observed, in particular that of cytotoxic T cells, suggested that mice immunized in such a way should be protected from a lethal dose of *L. monocytogenes.* Therefore, 90 days after the first immunization or 48 days after the fourth immunization - where applicable - mice were challenged i. v. with a dose of bacteria corresponding to 10 x LD_{50}. Fig. 4 shows that animals which were immunized four times with *Salmonellae* harboring an eukaryotic expression vector that encodes truncated LLO were completely protected (Fig. 4A). Animals that had received a single vaccination only were partially but significantly protected. At the time of termination of the experiment 60 % of the animals were still alive. All animals that were immunized with *Salmonellae* that carried the β-gal control plasmid were not protected and died within four days. Surprisingly, immunizations with *Salmonellae* carrying the ActA expression plasmid did not result in protection, although strong cytotoxic and helper T cell responses could be demonstrated in mice from the same group indicating that the immunization had been successfull (data not shown). Thus the membrane protein ActA is not a protective antigen.

Evidence for transfer of the expression plasmid from the carrier Salmonellae to host cells in vivo

We were concerned that a weak activity of the eukaryotic promoter in the bacteria or a cryptic prokaryotic promoter in the plasmid could result in expression of the antigens in the bacterial carrier thus eliciting the potent immune response. In fact, in the recombinant *Salmonellae*

haboring the pCMVβ weak β-gal activity (2.5 U) could be demonstrated. To exclude this possibility, we immunized mice with a *Salmonella* recombinant that contained more then 100 fold higher levels (334 U) of β-gal enzymatic activity. With a single vaccination using the latter bacteria no messurable T cell or antibody response was detected (Fig. 5A-C). Repeated vaccination, however, resulted in a weak cytotoxic T cell response detectable after *in vitro* restimulation, although, it barely reached the strength of the response observed using a single immunization with *Salmonellae* harboring the eukaryotic expression plasmid of β-gal (Fig. 5A). Neither a CD4 T cell nor an antibody response was observed even after repeated oral immunization with *Salmonellae* constitutively expressing β-gal (Fig.5B and C).

As a result of the *aroA* mutation bacteria die very quickly since live bacteria could never be demonstrated after immunization at various time points examined. Nevertheless, even five weeks after oral application of *Salmonellae* harboring the eukaryotic β-gal expression plasmid, enzymatic activity of β-gal could be detected in adherent cells - most likely macrophages - from the spleen of these mice (data not shown). To further corroborate this observation we injected *Salmonellae* carrying the pCMVβ vector into the peritoneum of mice and harvested the peritoneal exudate cells after 1 hour. Cells were then cultured overnight in the presence of tetracycline to inhibit bacterial protein synthesis and finally stained for β-gal activity. Enzymatic activity of β-gal was observed in a large number of macrophage like cells. The staining was diffuse and clearly not restricted to the endocytic vesicles in which *Salmonella* usually reside. This suggests that plasmid DNA was transferred from dying *Salmonellae* to host cells and occured with a high frequency *in vivo*.

DNA transfer from S. typhimurium aroA to mammalian host cells in vitro

To obtain direct evidence that DNA transfer from the bacterial carrier to the mouse macrophages can take place, primary peritoneal macrophages were infected with *Salmonellae* harboring the β-gal expression plasmid (pCMVβ). After infection for one hour, gentamicin was added to kill remaining extracellular bacteria. Four hours later tetracycline was added to kill the intracellular bacteria. This second antibiotic was later found to be unnecessary because strains harboring the *aroA* mutation survive only for brief periods of time in these cells (data not

shown). After overnight incubation, cells were stained for activity of β-gal. In up to 30 % of the adherent, macrophage-like cells, enzymatic activity could be demonstrated even in the continuous presence of tetracycline which blocks bacterial protein synthesis (Fig. 5).

To show that β-galactosidase was produced by the host cell, and not by the bacteria, two type of experiments were performed. Firstly, adherent peritoneal cells were infected and treated as described above. After overnight incubation RNA was extracted. If the plasmid was tranferred and transcribed in the nucleus of the host cell, RNA splice products derived from the splice donor and acceptors within the vector should be demonstratable. By RT-PCR with a primer pair that hybridises to sequences on either side of the small intron, a PCR product could be observed which corresponds to one of the expected splice products (Fig. 6A). The identity of this product was confirmed by DNA sequencing (data not shown). Secondly, biosynthetic labelling of proteins in the presence of tetracycline should only allow translation of mRNA produced by the eukaryotic host cells. Adherent peritoneal cells were infected as described and were pulsed for 30 min with ^{35}S-methionine after 4, 24 or 48 hours in the absence or presence of tetracycline. At four hours no β-gal could be observed by immunoprecipitation, not even in the absence of tetracycline where bacterial products should have been labelled (Fig. 6B). Thus transfer of plasmid DNA and eukaryotic expression had not taken place yet. However, β-gal could be immunoprecipitated following a 24 hour or 48 hour incubation period even when tetracycline was continuously present during incubation and labelling period. Preincubation of the anti-β-gal antibody with an excess of unlabeled β-gal demonstrated the specificity of the immunoprecipitation (Fig. 6B/lane 10). This clearly indicates that the β-gal precipitated was produced by the infected mammalian host cell itself and not by the bacterium which had originally carried the expression plasmid. Thus, a transfer of the plasmid from *Salmonellae* to the host cell must have taken place.

Discussion

The transfer of eukaryotic expression plasmids from attenuated enteric bacteria into the nucleus of host cells has recently been demonstrated. While this work was in progress it was reported that attenuated *Shigella* and *E. coli* that express the invasin of *Shigella* can carry eukaryotic expression plasmids into host cells. The plasmids are transferred into the nucleus and expressed upon death of the bacteria. Both bacteria escape from the phagolysome into the cytosol of the host cell. The transfer of plasmid DNA can thus easily be explained by lysis of the bacteria which subsequently allows its liberation and transfer into the nucleus. The fact that DNA transfer is also possible with attenuated *Salmonella*, on the other hand, was unexpected. *Salmonellae* usually do not escape from the phagolysosome but rather remain in vesicular compartments. However, it has been described that some cell types e. g. macrophages possess a pathway that permit transfer of proteins from endocytic vesicles into the cytosol. Whether such a pathway could also be responsible for the transfer of nucleic acids remains to be studied. Plasmid transfer with *Salmonella* was only observed with primary macrophages and not seen with several macrophage cell lines (data not shown). This suggests the presence of a transport system which is operating efficiently only in the primary cells.

Evidence for a transfer of plasmid DNA from *Salmonella* to the host cell *in vitro* is compelling. Splicing of RNA and protein synthesis in the presence of tetracycline are both only possible if the gene is expressed by the eukaryotic host cell. Evidence that a transfer of the expression vector *in vivo* is responsible for induction of the strong immune response observed, also was obtained. Enzymatic activity of β-gal could be observed five weeks after the last challenge in a few adherent spleen cells.However, viable *Salmonella* could not be detected even when tested one week after the last infection, thus, arguing that β-gal expression cannot be due to residual surviving *Salmonella*. How antigen expressing cells can coexist with specific cytotoxic T cells is intriguing and will need further experimentations.

Strong cytotoxic and protective responses have only been reported with *Salmonella* that secrete the antigens. No comparable responses have been described using *Salmonella* that constitutively express nonsecreted heterologous proteins. Bacterial doses at least 50 times higher than the dose

used in this study were needed to induce CD8 T cells. No antibody response was observed under these circumstances. This was confirmed by our own results (Fig. 5). We, therefore, find it highly unlikely that the strong responses of cytotoxic and helper T cells as well as the specific antibody production is due to a fortuitous expression of the antigens in the *Salmonella* carrier.

The strength of the immune response observed especially after a single dose of immunization indicates that transfer of DNA by bacterial carrier is probably superior to a direct application of isolated plasmid DNA into skin or muscles. This suggests that by using the natural port of entry of a pathogen, the expression vector is transferred into cell types that have evolved to efficiently induce an immune response. It is likely that the *Salmonella* carrier is taken up by macrophages and dendritic cells. Whether, macrophages play a role during stimulation of naive T cells against bacteria is not clear, but dendritic cells are known to be highly efficient in inducing resting T cells. Since the antigen is expressed in the cytosol of these cells a strong cytotoxic T cell response is to be expected. Why an additional strong helper and antibody response is taking place, can only be speculated. Some cytosolic proteins can efficiently be presented by MHC class II molecules. However, it would be a very fortunate coincidence if all three proteins used in the present study display this property. It is more likely that APC expressing the antigen are lysed by specific cytotoxic cells and dying cells or free antigen is taken up by neighbouring APC and presented via MHC class II molecules. The humoral response could be explained in a similar way.

In summary, oral genetic immunization using attenuated *Salmonellae* as carrier could work as schematically depicted in Fig. 7. *Salmonella* enter the host via M cells in the intestine. The bacteria are taken up in the dome areas by phagocytic cells such as macrophages and dendritic cells. These cells are activated by the pathogen and start to differentiate and probably to migrate into lymph nodes and spleen. During this time period the bacteria die due to their attenuating mutation and liberate the eukaryotic expression vectors. The plasmids are then transferred into the cytosol either via a specific transport system or by endosomal leackage. Finally, the vector enters the nucleus and is transcribed, thus, leading to antigen expression in the cytosol of the host cells. Specific cytotoxic T cells are induced by these activated APC which lyse antigen expressing cells. Free antigen or dying cells can be taken up by other APC, which now in turn

can stimulate helper cells. Free antigen also is responsible for the induction of an antibody response. In addition, bacterial endotoxin and DNA sequence motifs of the vector could also function as adjuvant and could contribute to the strong responses observed.

The helper T cell response induced with this type of genetic immunization seemed strongly biased to the TH1 type as indicated by IFNγ production of restimulated T cells *in vitro* and the high titer of IgG2a in the humoral response. This is not unexpected since bacteria usually induce inflammatory type of responses. For many vaccination strategies it is desirable to induce an TH1 response for protection against the particular pathogen, e. g. strains of mice which respond with TH2 cells against *Leishmania major* cannot clear the parasite and are not protected, while mice which mount a TH1 response are resistant. On the other hand, induction of TH2 type of responses or the conversion of a TH1 response into a TH2 response has been shown to be advantageous in inflammatory autoimmune diseases. Similarly, infections by nematodes might also require a TH2 response. Since the bacteria only being used as a vehicle in transferring the expression plasmids and therefore play only a secondary role it should be possible to manipulate the TH1 response. The induction of specific IgG1 suggests a TH2 component during the helper response that might be augmentable. Co-expression of the antigen together with certain cytokines or costimulatory molecules or alternatively using antisense strategies to suppress costimulatory molecules should make it possible to drive the responses more towards TH2.

Two well characterized virulence factors were tested as antigens for protection against a lethal challenge with *L. monocytogenes*. Listeriolysin has been shown before to induce protection. This was also true under our experimental conditions. Interestingly, even a single dose of *Salmonellae* harboring the eukaryotic listeriolysin expression plasmid was sufficient to afford protection to 60 % of the mice. ActA cannot serve as protective antigen. The membrane protein ActA obviously is not available to the presentation mechanisms as long as the bacteria are alive. This raises the question as to whether membrane proteins of bacteria in general are not protective or whether ActA is a special case. Extensive phosphorylation of the ActA protein by host kinases following infection may affect its ability to be processed. Nevertheless, the role of bacterial surface-bound proteins in protection can now easily be addressed using the *Salmonellae* system for genetic vaccination.

The induction of a strong and specific antibody response which can be messured in ELISA and by immunoblot revealed additional benefits derived from the type of immunization described here. Thus, to raise specific polyclonal and possibly also monoclonal antibodies, any open reading frame can be inserted into an expression plasmid and used for immunization. This will facilitate the characterization of gene products where only sequence information is available.

In conclusion: using attenuated *Salmonella* which carry eukaryotic expression vectors, genetic immunization can be achieved by oral administration of the carrier. The stimulation of cytotoxic and helper T cells as well as the induction of a strong antibody response provides a very versatile system for new immunization strategies. The strength of this approach also draws on the development of newer more rationally attenuated *Salmonellae* strains as well as technical advances in providing conditional and targeted eukaryotic expression by the infected host cell. The possibility of genetic immunization with DNA fragments containing open reading frames will allow to define the function of new gene products, provide serological reagents, and protective antigens for vaccination.

Experimental procedures

Mice

Female BALB/c (H-2^d) mice 6-8 weeks old, were obtained from Harlan Winkelmann (Borchem, Germany).

Media, reagents and antigens

RPMI (Gibco), supplemented with 10 % fetal bovine serum, where required, was used as culture medium for eukaryotic cells and all functional assays were performed in this medium. Solid and liquid Luria Bertani medium (LB, Sambrook) was used for growing *E. coli* and *S. typhimurium* strains. Brain heart infusion broth or agar (BHI; Difco, Detroit, USA) was used for growing *L. monocytogenes* EGD. Media were supplemented, where required with 100 µg/ml of ampicillin. Concanavalin-A (con-A), hen egg lysozyme (HEL), tetracycline, β-galactosidase of *E. coli.* potasium ferrocynide and potasium ferricynide were purchased from Sigma (Sigma, St. Louis, USA), listeriolysin was purified as described ( ). Soluble ActA protein (AA 31-505) was purified from supermutants of recombinant *L. monocytogenes* (Gerstel et al. to be published).

Bacterial strains and plasmids

The *E. coli* strain XL1-blue (Strategene, Heidelberg, Germany) was used as a host during the cloning experiments and to propagate plasmids. The auxotrophic *S. typhimurium aroA* strain SL7207 (*S. typhimurium 2337-65* derivative *hisG*46, DEL407 [*aroA*::Tn10{Tc-s}]) was used as a carrier for the *in vivo* studies. The hemolytic *L. monocytogenes* strain EGD (serotype 1/2a; Chakraborty 1992) was used for *in vivo* protection assays and preparation of genomic DNA. The DNA was used as template for the PCR amplification of *actA* and *hly* genes. The eukaryotic expression vector pCMV-β (Clontech, Palo Alto, USA) containing β-gal of *E. coli* was used for the cloning by replacing β-gal with the amplification products containing a truncated variant of *actA* or of *hly*. For expression of β-gal in *Salmonellae* the plasmid pAH97 was used. It contains the Pr and Ps promotor of the *Xyl S* gene of *Pseudomanas putida* and results in constitutive expression of β-gal (384 U) in the *S. typhimurium aro A* strain. Bacterial cultures were grown at 37°C and aerated by shaking at 200 r.p.m.

Recombinant DNA techniques

DNA preparation, genetic manipulations and PCR were carried out according to standard protocols (Sambrook), and plasmid DNA transformation of bacterial cells was performed as described by Hannahan or by electroporation (O'Callaghan). DNA sequencing was performed using a Taq Dye Deoxy terminator cycle sequencing system (Applied Biosystems) and analyzed on an Applied Biosystems 373A automated DNA sequencer.

Cloning of actA and hly into the eukaryotic expression vector pCMV-β

For the construction of the eukaryotic expression vector pCMVActA, a 1.8 kb fragment encoding AA 31 to 613 of a ActA polypeptide without the membrane anchor (Domann 1992) was amplified by PCR using the forward and reverse primers:

5'-ATAAGAATGCGGCCGCCATGGCGACAGATAGCGAAGATTCTAGTC-3' and

5'-ATAAGAATGCGGCCGCTTACGTCGTATGGTTCCCTGGTTCTTC-3'; and genomic DNA from *L. monocytogenes* strain EGD as template. In a similar way recombinant plasmid pCMVhly was constructed. A 1.4 kb fragment encoding a non hemolytic variant comprising amino acids 26 to 482 of *hly* and deleting the peptide essential for hemolytic activity ( ) (Mengaud) was amplified using the forward and reverse primers:

5'-ATAAGAATGCGGCCGCCATGGATGCATCTGCATTCAATAAAGAAAATTC-3'and 5'-ATAAGAATGCGGCCGCTTATTTAGCGTAAACATTAATATTTCTCGCG-3'. PCR primers were designed in such a way that the resulting fragments contain *Not*I flanking restriction sites (underlined) and start and stop codons were introduced (ATG and TTA in bold). The PCR fragments were digested with NotI and ligated with NotI-digested pCMVβ, thereby generating pCMVActA and pCMVhly, respectively. The coding region for β-gal is deleted from pCMVβ by the NotI digestion . The DNA sequence of the inserted PCR fragments were verified by Taq Dye Deoxy terminator cycle sequencing.

Immunization and challenge

For immunization, groups of 5-10 female BALB/c mice were fed with 30 µl of 10% sodium bicarbonate buffer containing 10^8 recombinant *S. typhimurium aroA* strain harboring one of the eukaryotic expression vectors pCMVβ, pCMVhly or pCMVActA or the prokaryotic β-gal expression plasmid pAH97 ( ). Mice received either a single immunization or four

immunisations at 14 days intervals. Serum samples from both groups of mice were obtained on day -1, 7, 21, 35 and 63 and were stored at -20°C until used in enzyme-linked immunosorbent assay (ELISA) or immunoblot. Mice of each group were sacrificed at weeks 3, 5, 7 and 11 after the first immunization and tested for T cell responses. For protection studies, immunized mice were challenged *i.v.* on day 90 (one and half month following the last boost of mice receiving multiple immunizations) with a lethal dose of 5 x 10^4 *L. monocytogenes* EGD. Survival of mice was followed until day 14 post-challenge.

CTL assay

For the determination of induction of cytotoxic T cells the JAM assay was performed (Matzinger P.,1991). Briefly, 3 x 10^5 target cells were incubated for 4 h with 5 µCi ^{3}H-thymidine (Amersham), washed and co-cultured with the spleen cells isolated from mice immunized with *S. typhimurium aroA* strains, harboring the eukaryotic or prokaryotic expression vectors at different effector to target ratios. Spleen cells were either assayed straight *ex vivo* or after *in vitro* restimulation for 5 days. To test for LLO specific cytotoxic T cells, P815 target cells were sensitized with 1 µg/ml of LLO peptide AA 91-99. The ActA specific cytotoxicity was revealed by sensitizing the radiolabeled P815 cells with a mixture of 1µg/ml purified hemolytically active LLO and 1µg/ml of purified ActA protein for 30 min at RT. We have shown previously that it is possible to sensitize target cells very efficiently *in vitro* with soluble proteins by using the pore-forming activity of LLO.Target cells sensitized with LLO only were not lysed when T cells from mice that were immunized with *Salmonella* carrying the ActA expression plasmid were tested. This indicates that the assay is specific for ActA when mice were immunized with *Salmonellae* harboring ActA expression plasmids. To messure the β-gal specific cytotoxicity, P13.1 - a P815 derivative transfected with the β-gal gene - was used as target cells. Mixtures of effector and target cells were incubated for 4-5 h at 37°, then plates were harvested on filtermats which finally were counted in a scintillation counter. All assays were performed in triplicates in 200 µl final volume in round bottom 96 well microtiter plates

Proliferation assay

Induction of T helper cells was assayed by direct proliferation of cells isolated from spleens or lymph nodes of mice immunized with *S. typhimurium aroA* strains, harboring the eukaryotic

expression vector pCMVβ , pCMVhly or pCMVActA or the prokaryotic β-gal expression vector pAH97. Proliferation of T cells were directly analysed by ^{3}H-thymidine incorporation. Briefly, 2 x 10^{5} T cells were co-cultured with 1 x 10^{5} irradiated syngeneic spleen cells together with either 0,5 µg/ml purified LLO, 1 µg/ml Act-A or 1 µg/ml β-gal. After 48 h of incubation at 37°C, 1µCi ^{3}H-thymidine was added to each culture and after further 18 h of incubation, cells were harvested on filter mats and incorporation was counted in a scintillation counter. All experiments were performed in triplicates in 200 µl final volume in flat bottom 96 well microtiter plates.

ELISA

To evaluate the levels of immunoglobulins against LLO, Act-A and β-gal in serum specimens, 96-well ELISA plates (Maxisorp, Nunc) were coated with 0.5 µg/ml purified protein overnight at 4°C. Plates were washed three times with PBS/0.05% Tween 20 and then blocked with 3% BSA-PBS for 2 h at 37°C. Following two washes with PBS/0.05% Tween 20, serum samples of a 1:100 dilution were added to individual wells and incubated for 2-3 h at 37°C. Plates were washed as above and biotinylated goat anti-mouse Ig (Dianova, Hamburg, Germany) in 1% BSA-PBS was added to each well and incubated for 1 h at 37°C. After three washes with PBS/0.05% Tween 20, horseradish peroxidase conjugated streptavidin (Dianova, Hamburg, Germany) in 1% BSA-PBS was added to each well and incubated for 1 h at 37°C. Plates were washed as above, developed with *o*-Phenylene diamine (OPD) as substrate and messured in an ELISA reader at 490 nm. For antigen specific IgG subclasses determination, peroxidase conjugated goat anti mouse IgG1, IgG2a, IgG2b and IgG3 (Caltag laboratories, CA, USA) were used.

Detection of β-gal activity

Expression of β-gal in host cells was monitored by incubating the fixed cells with the indicator substrate X-gal. Briefly, isolated peritoneal macrophages were allowed to adhere for a couple of hours at 37°C in 24 well-plate in antibiotic free medium. After removing the non-adherent cells and washing with antibiotic free medium, *S. typhimurium aroA*, harboring the eukaryotic expression vector pCMVβ were added to the cells at a MOI of 10 and incubated at 37°C for 15-30 min. Cells were washed again and bacteria remaining extracellular were killed by addition of

medium containing 50 µg/ml gentamicin. Following 4 h of incubation at 37°C, 10 µg/ml of tetracycline was added to some of the cultures to block the intracellular bacterial multiplication and incubation was continued for further 24 h. After 2-3 washes with PBS, cells were fixed with acetone/methanol (1:1 v/v) and freshly prepared X-gal substrate (5 mM potasium ferrocynide, 5 mM potasium ferricynide, 2 mM $MgCl_2$ and 100 µg/ml X-gal in PBS) was added. After overnight incubation at 37°C β-gal expressing cells were detected by light microscopy. Quantitation of β-gal enzymatic activity in recombinant bacteria was determined according to standard procedure (Sambrook et al.).

RNA isolation and RT-PCR

In order to test for expression of β-gal transferred into the eukaryotic host cells via *Salmonella,* the m-RNA was probed for the presence of splice products derived from the splice donor and acceptors of the expression plasmid. To this end, PECs were infected *in vitro* at a MOI of 10 with *S. typhimurium aroA* harboring the eukaryotic expression vector pCMVβ and RNA was extracted as described (Chomczynski and Sacchi, 1987). RT-PCR of isolated RNA was performed according to published procedures (???). Briefly, 10 µg of isolated total cellular RNA was resuspended in 20 µl of DEPC-H_2O and incubated for 5 min at 70 °C with 10 µl of buffer containing 6 µl of reverse transcriptase buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$); 0,4 mM dNTPs; 0,05 U random hexamers (Pharmicia, Uppsala, Sweden); and 1 mM DTT. Samples were spun down for 2 min at 15,000 rpm and 40 U RNAsin ribonuclease inhibitor (Promega) together with 200 U Superscript reverse transcriptase (Gibco, BRL) were added. RNA was reverse transcribed for 45 min at 37°C and the reaction was stopped by heating the samples at 95°C for 1 min followed by a short incubation on ice. Subsequently 500 ng of cDNA product was amplified by PCR in a final volume of 50 µl containing 0,2 mM dNTP, 20 mM DTT, 3 µM of each of the 5´and 3´ primer, 5 µl of 10x PCR buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 1% gelatine, 1,5 mM $MgCl_2$, 1% Triton X-100) and 5 U AmpliTaq-DNA-polymerase (Perkin Elmer). PCR was performed with an initial denaturation step of 10 min at 85°C followed by 35 cycles of 20 sec denaturation at 95°C, 30 sec annealing at 60°C and 30 sec extension at 72°C. The amplification products were visualized under an UV lamp after electrophoresis of a 15 µl aliqot of the reaction mixture on a 2% (w/v) agarose gel containing 0,5 µg/ml of ethdium bromide. The primer pair was designed in such a way that the

presence of splice products should be indicated by a 190bp and/or a 125bp fragment. The identity of the presumable splice product was confirmed by sequencing the fragments after isolation on a preparative agarose gel. The primer pair used for amplification and sequencing - SV40 forward: 5′-GGATCCGGTACTCGAGGAAC-3′,SV40 reverse : 5′-GCTTTAGCAGGCTCTTTCG-3′.

Immunoprecipitations

Biosynthetic labeling of proteins in the presence of tetracycline followed by immuneprecipitation should only reveal protein expression by eukaryotic host cells. Therefore, 5 x 10^5 adherant PECs were infected for 30 min at 37°C with ca. 5 x 10^6 *S. typhimurium aroA* harboring the eukaryotic expression vector pCMVβ in antibiotic free medium. After a thorough wash and further 4 h of incubation at 37°C, medium was supplemented with antibiotics or not and left at 37°C for various periods of time before biosynthetic labeling. After two washes in PBS and 30 min starvation in methionine-free medium, cells were pulsed with 100 µCi [^{35}S]methionine for 2 h . Then cells were carefuly washed and lysed in 0.5 ml ice cold lysis buffer (0.5% NP-40, 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM PMSF). After 45 min on ice, cells were centrifuged to remove nuclei and cell debris, and incubated at 4°C with 4 µg of anti-β-gal antibodies for 30 min. Immune complexes were precipitated with protein A sepharose in 0.5% NP-40, 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$ and 0,5mM NaCl washed several times with the same buffer and analysed on 8% SDS-PAGE followed by fluorography. Into some samples a 100-fold excess of β-gal protein was added before addition of anti-β-gal antibodies to determine the specificity of the precipitation.

Legends to Figures:

Fig. 1

Induction of cytotoxic T cells in mice orally immunized with 10^8 *S. typhimurium aroA* carrying eukaryotic expression plasmids which encode listeriolysin or ActA. Mice were immunized either four times with two week intervalls (A, B, D, E) or once (C; F) with *Salmonella* carrying pCMVhly (A-C) or pCMVActA (D-F) and spleen cells were restimulated once *in vitro* with a synthetic peptide comprising AA91-99 of listeriolysin (A-C) or with a mixture of purified ActA and hemolytically active listeriolysin which results in the class I presentation of ActA due to the pore-forming activity of listeriolysin ( ). Restimulated T cells were tested with radiolabelled P815 target cells at an effector to target ratio of 10:1. A: Specificity of the anti-listeriolysin cytotoxic response. Target cells were sensitized with henegglysozyme (HEL), peptide AA 91-99 of listeriolysin (pLLO) or control peptide of nucleoprotein of influenza virus (pNP). Displayed is the experiment with spleen cells from week 5 shown in panel B. Similar specificity was observed at all other time points. B: Kinetic of the cytotoxic response of mice immunized four times with pCMVhly. The arrows indicate the booster immunizations. C: Kinetic of the cytotoxic response of mice immunized once with pCMVhly. D: Specificity of the anti-ActA cytotoxic response. Target cells were sensitized with a mixture of ActA and listeriolysin (ActA + LLO), hen egg lysozyme and listeriolysin (HEL + LLO) or listeriolysin alone (LLO). Displayed is the experiment with restimulated spleen cells from week 5 shown in panel E. Similar specificity was observed at other time points and including other synthetic peptides of various sources. E: Kinetic of the cytotoxic T cell response in mice immunized four times pCMVActA. Arrows indicate booster immunisations. F: Kinetic of the cytotoxic T cell response in mice immunized once with pCMVActA. The specificity of the cytotoxic response was further assed by testing the spleen cells of mice immunized in a similar way with pCMVβ (β-gal) on target cells sensitized with pLLO, ActA plus listeriolysin or a β-gal expressing transfectant of P815 (data not shown). Similarly, a specific cytotoxic T cell response was observed against β-gal, but the kinetic was not followed as systematically as for the two other antigens.

Fig. 2

Induction of helper T cells in mice orally immunized with 10^8 *S. typhimurium aroA* carrying eukaryotic expression plasmids which encode listeriolysin or ActA. Spleen (SPC) and lymphnode cells (LNC) from the same mice tested for cytotoxic T cell responses displayed in Fig. 1 were tested for T helper responses. Mice were immunized either four times (A, B, D, E) or once (C; F) with *Salmonella* carrying pCMVhly (A-C) or pCMVActA (D, F) and restimulated *in vitro*. After two days proliferation was tested by incorporation of ^{3}H-Thymidine. **A**: Specificity of the proliferative response of spleen cells from mice immunized with pCMVhly. T cells tested were the same as those displayed in Panel B at week 11. Similar results were obtained at other time points. **B**: Kinetic of the proliferative response of spleen and lymph node cells from mice immunized four times with pCMVhly. Arrows indicate the booster immunizations. **C**: Kinetic of proliferative response of spleen and lymph node cells from mice immunized once with pCMVhly. **D**: Specificity of proliferative response of spleen cells from mice immunized four times with pCMVActA. T cells tested were the same as those displayed in Panel D at week 11. Similar results were obtained at other time points. **E**: Kinetic of the proliferative response of spleen and lymph node cells immunized four times with pCMVActA. Arrows indicate booster immunizations. F: Kinetic of the proliferative response of spleen and lymph node cells from mice immunized once with pCMVActA. Similarly, spleen and lymph node cells from mice immunized with pCMVβ (β-gal) never reacted with either listeriolysin or ActA but could respond to restimmulation with β-gal (data not shown).

Fig. 3

Kinetic and subclass distribution of specific serum IgG from mice orally immunized with *S. typhimurium aroA* carrying eukaryotic expression plasmids which encode listeriolysin, ActA or β-gal. Sera from the same mice tested for cytotoxic and proliferative T cell responses displayed in Fig. 1 and 2 were used and assayed in specific ELISA's. Mice were immunized four times (A) or once (B) with pCMVhly, pCMVActA or pCMVβ respectively, and pooled sera were tested for antigen specific serum IgG. To asses specificity all sera were tested on all three antigens. Reactivity was only observed against the immunizing antigen (data not shown). Identical results were obtained by immunoblotting usig the same antigens (data not shown). The subclass distribution 11 weeks after the first immunization was determined from the sera of individual mice immunized four times (closed symbols) or once (open symbols) with either

pCMVhly (C) or pCMVActA (D).

Fig. 4

Oral immunization with *S. typhimurium aroA* carrying the eukaryotic expression plasmid which encodes listeriolysin induces a protective immune response while immunisation with bacteria carrying the expression plasmid for ActA is not protective. Groups of six mice were immunized four times with two week intervalls (A) or only once (B) with *Salmonella* carrying pCMVhly, pCMVActA or pCMVβ and challenged with a lethal dose of 5×10^4 *L. monocytogenes* EGD (10 x LD_{50}) intravenously. Mice that had been immunized only once with pCMVhly became moribund after two days. However, four of them recovered and survived in good condition until the experiment was terminated after two weeks later.

Fig. 5

Comparison of orally induced immune responses elicited with *Salmonella* haboring prokaryotic or eukaryotic expression plasmids for β-galactosidase. Mice were immunized with *Salmonella* haboring either the eukaryotic expression plasmid pCMVβ or the plasmid pAH97 that constitutively expressed β-gal from the Pr and Ps promotor of *XylS* of *Pseudomonas putida*. Bacteria haboring the eukaryotic vector were administered orally once (●) while bacteria expressing β-gal under the control of the prokaryotic promotor were administered either once (◆) or four times with two week intervals (▼). The arrows indicate the time of booster immunisations. A. Cytotoxic response of restimulated spleen cell tested at an effector to target ratio of 10:1. The β- gal expressing transfectant P13.1 was used as target in the JAM assay. B. Proliferative helper T cell response of spleen cells with isolated β-gal as antigen. C. Antibody response against β-gal from pooled sera messured by ELISA. Data displayed in A-C were obtained with cells or sera from the same mice. All assays were performed as described in figures 1-3.

Fig. 6

Expression of enzymatic activity of β-galactosidase in peritoneal exudate cells after infection with *S. typhimurium aroA* that harbor an eukaryotic expression plasmid for β-gal. Freshly isolated peritoneal exudate cells (PECs) were allowed to adhere for two hours and infected at a MOI of 10 for 15 min with *Salmonella* bearing pCMVβ in antibiotic free medium. Following a

wash and addition of gentamycin to kill bacteria which remained outside of the cells, incubation was continued for 3-4 h at 37° C. Medium was then supplemented with tetracycline to kill the bacteria by blocking their protein synthesis. After additional 24 h at 37° C cells were washed with PBS, dried, fixed with acetone/methanol and incubated overnight with the X-gal substrate. Sometimes, expression of β-gal activity in up to 30 % of the adherent cell population was observed. Only macrophage-like cells expressed enzymatic activity. The small cells found in displayed cultures most likely represent nonadherent lymphocytes which were not removed in this particular experiment. Tetracycline remained in the medium through the whole experiment. Staining the cells already after 4 h did not reveal any enzymatic activity.

Fig. 7

The eukaryotic host cells transcribe and translate β-galactosidase derived from *S. typhimurium aroA* harboring the expression plasmid. A : RNA derived from PEC's 24 h after infection with *Salmonella* carrying pCMVβ was analysed by RT-PCR. A primer pair that borders the splice donor and acceptors sites downstream of the promotor was used. In lane 2 a band of 196 bp (indicated by the arrow) could be detected. DNA sequencing identified this fragment as a splice product. The stronger 227 bp long fragment seen in this lane is either due to carry over of DNA into the RNA preparation or due to inefficient splicing. Lane 1 shows the untreated macrophage control and the lane marked M contains the molecular size marker used. **B**: PEC's were infected with pCMV carrying *Salmonella* as described and after incubation at various length of time biosynthetic labelling was performed in the presence or absence of tetracycline followed by immunoprecipitation with β-gal specific monoclonal antibodies. Controls: (1) BHK cells; (2) BHK cells transfected with β-gal (positive control). Infected PEC's: (3) incubated four hours *post infection* (p.i.), without tetracycline; (4) incubated four hours p. i., with tetracycline during labelling; (5) incubated four hours p.i., with tetracycline during incubation and labelling; (6) incubated 24 h p.i., without tetracycline; (7) incubated 24 hours p.i., with tetracycline during labelling; (8) incubated 24 h p.i., with tetracycline during incubation and labelling (9) incubated 48 h p.i., without tetracycline; (10) incubated 48 h p.i., with tetracycline during labelling, a 100 fold excess of β-gal over the precipitating antibody was added to the lysate before immunoprecipitation; (11) incubation of 48 h p.i., with tetracycline during labelling; (12) incubation 48 h p.i., with tetracycline during incubation and labelling. No specific band was

observed after 4 h of incubation under any conditions. However, after allowing 24 h or more for a DNA transfer and expression to occur, a specific band for β-gal - indicated by the arrow - can be observed.

Fig. 8

Model of oral genetic immunization with attenuated *S. typhimurium aroA*.

April 17, 1997/hl

Our ref.: 8579
New European Patent Application
Gesellschaft fuer Biotechnologische Forschung mbH (GBF)
Weiß et al.; *Salmonella typhimurium* strain

Patent Claims

1. Attenuated strain of *Salmonella typhimurium* carrying an eucaryotic expression vector containing the gene for a truncated form of listeriolysin (hly) controlled by an eucaryotic promoter.

2. Strain according to claim 1, wherein the strain is *S. typhimurium* aroA SL 7207.

3. Strain according to claim 1 or 2, wherein the strain is obtainable by the following measures:

(i) the known plasmid pCMVß containing the structural gene of ß-galactosidase (ß-gal) under the control of the human CMV early promoter and including a splice donor and two splice acceptor

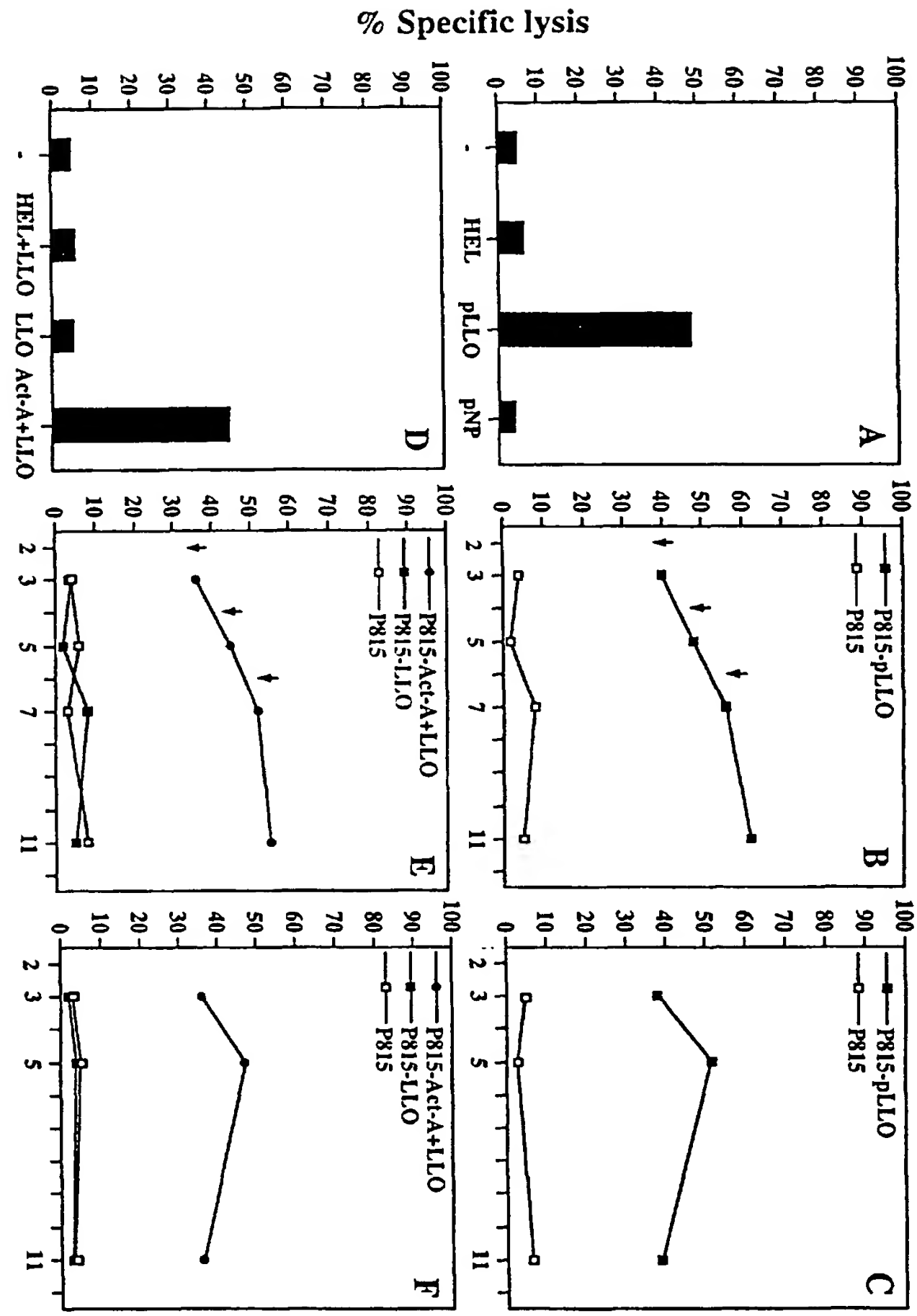
sites in between the promoter and the ß-gal gene, is used as starting eucaryotic expression vector and

(ii-a) the ß-gal gene is replaced by a structural gene encoding a non-hemolytic truncated variant of listeriolysin (hly) or

(ii-b) the ß-gal gene is replaced by a structural gene encoding a truncated variant of the membrane protein ActA and

(iii) the strain of *S. typhimurium* is transformed with the plasmid (pCMVhly) resulting from (ii-a) or with the plasmid (pCMV ActA) resulting from (ii-b).

Fig. 1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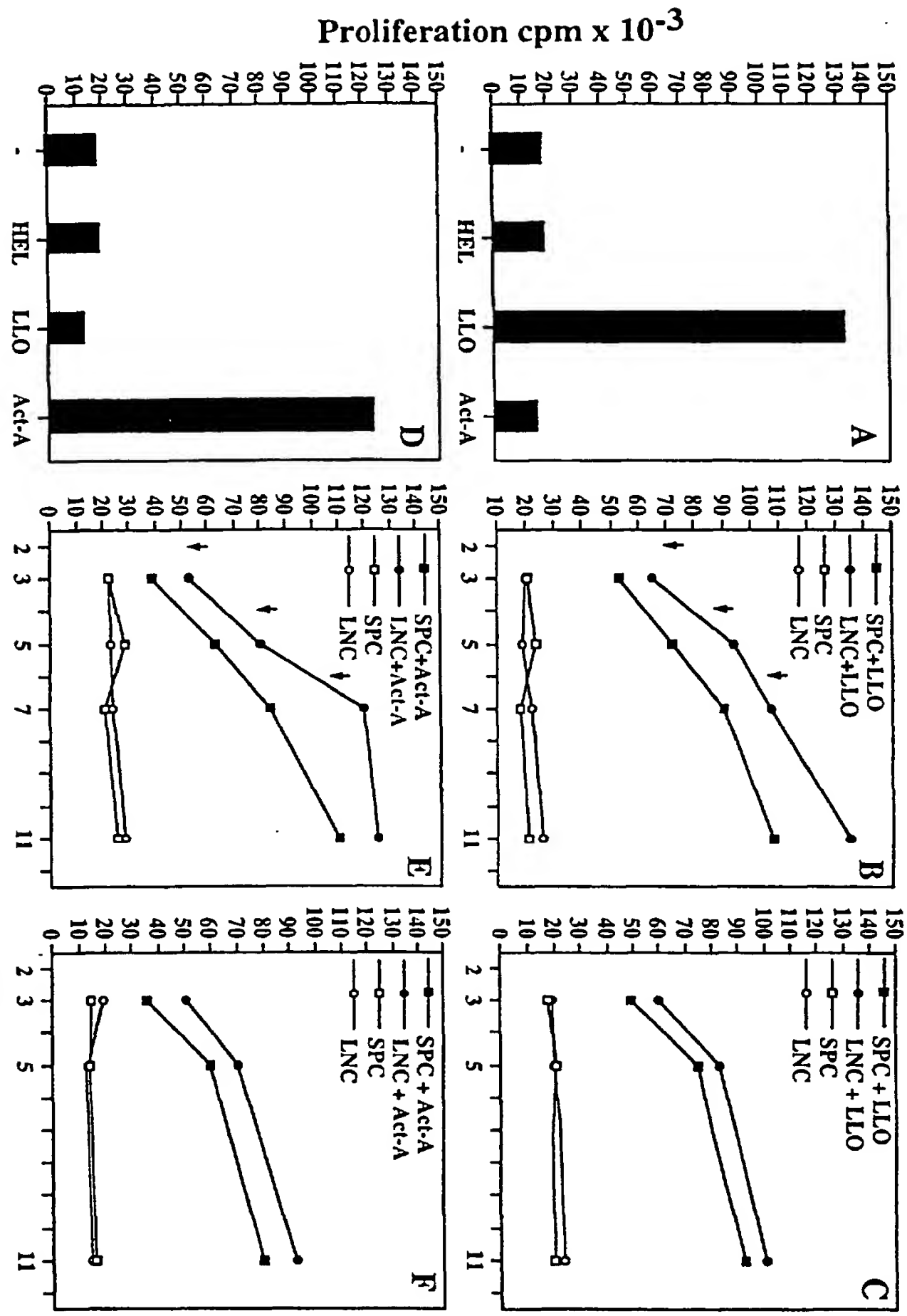
A
HEL
pLLO
pNP
B
C
D
HEL+LLO
LLO
Act-A+LLO
E
F
P815-pLLO
P815
P815-Act-A+LLO
P815-LLO
Time (Weeks)

Fig. 2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B
C
D
E
F
-
HEL
LLO
Act-A
SPC+LLO
LNC+LLO
SPC
LNC
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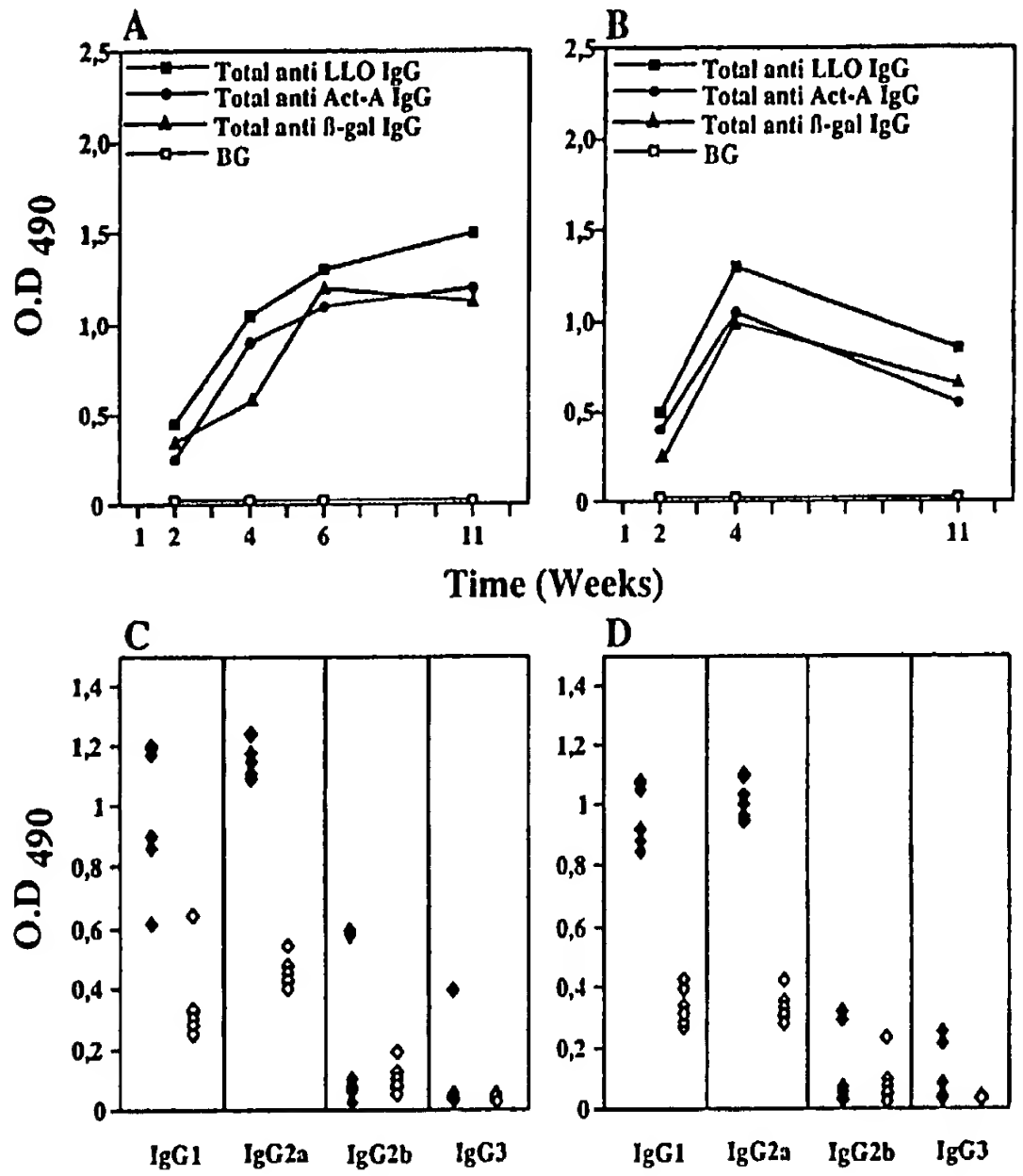
LNC+Act-A
SPC + LLO
LNC + LLO
SPC + Act-A
LNC + Act-A
Time (Weeks)


A
B
Total anti LLO IgG
Total anti Act-A IgG
Total anti ß-gal IgG
BG
O.D 490
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2,0
1,5
1,0
0,5
0
1 2 4 6 11
1 2 4 11
Time (Weeks)
C
D
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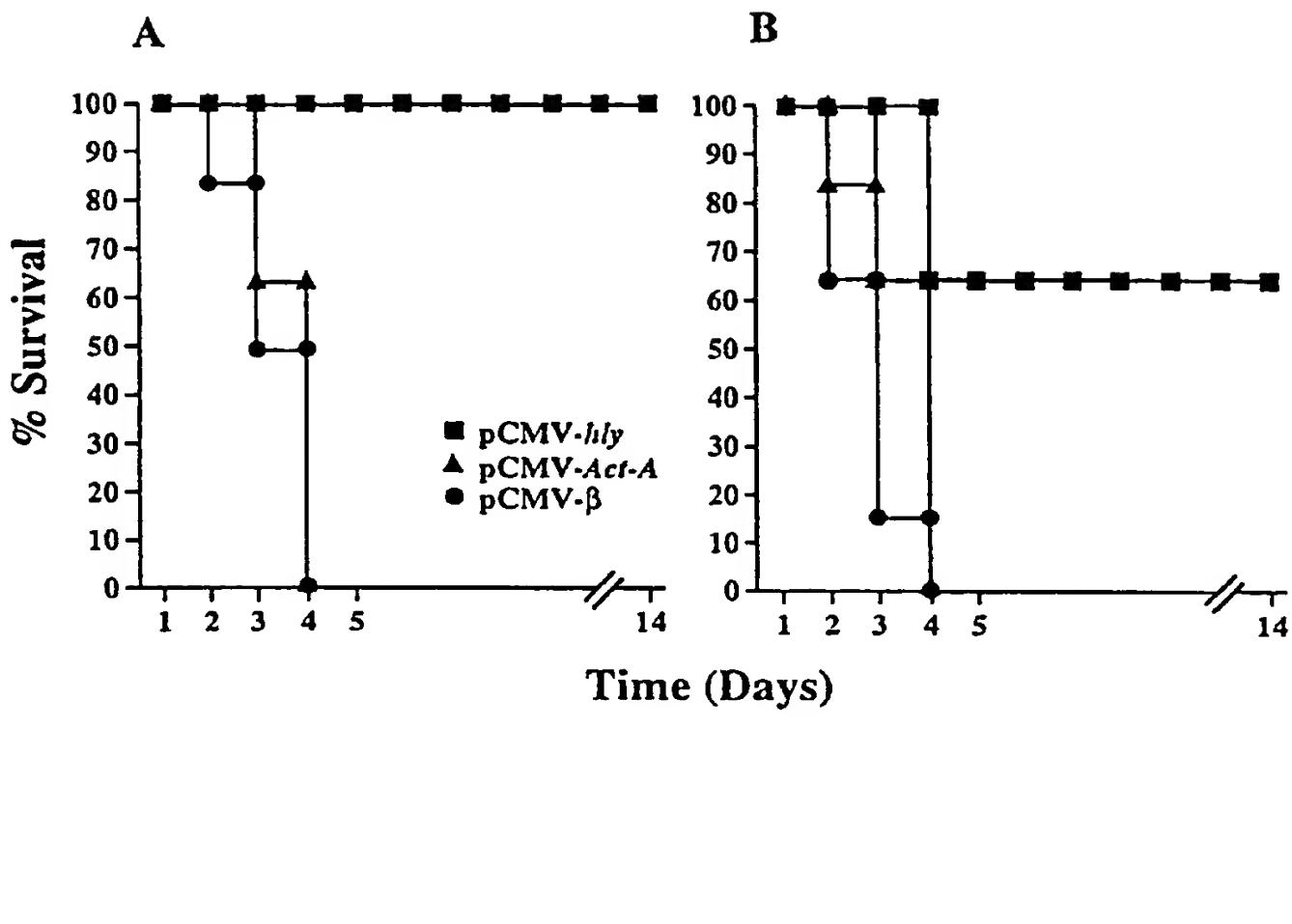
1,2
1
0,8
0,6
0,4
0,2
0
IgG1 IgG2a IgG2b IgG3

Fig. 3

Fig. 4


A
B
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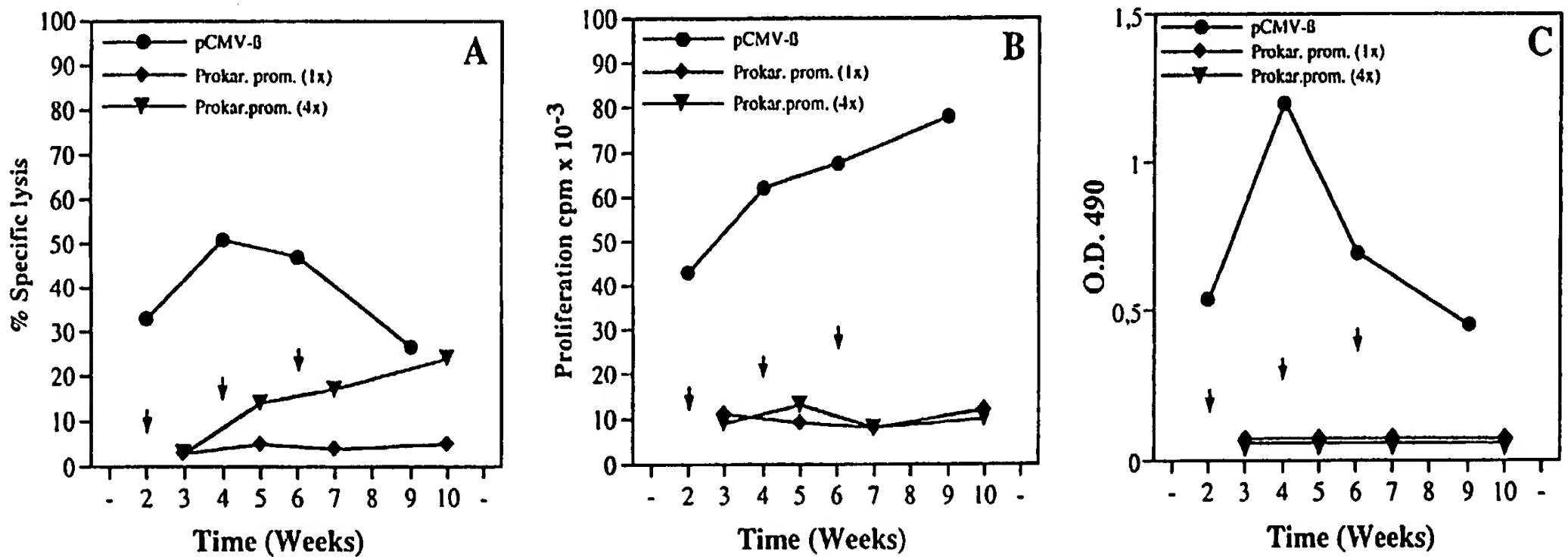
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
14
pCMV-*hly*
pCMV-*Act-A*
pCMV-β
Time (Days)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pCMV-ß
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Prokar.prom. (4x)
% Specific lysis
100
90
80
70
60
50
40
30
20
10
0
- 2 3 4 5 6 7 8 9 10 -
Time (Weeks)
B
pCMV-ß
Prokar. prom. (1x)
Prokar.prom. (4x)
Proliferation cpm x 10-3
100
90
80
70
60
50
40
30
20
10
0
- 2 3 4 5 6 7 8 9 10 -
Time (Weeks)
C
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Prokar. prom. (1x)
Prokar.prom. (4x)
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- 2 3 4 5 6 7 8 9 10 -
Time (Weeks)

Fig. 5

Fig. 6

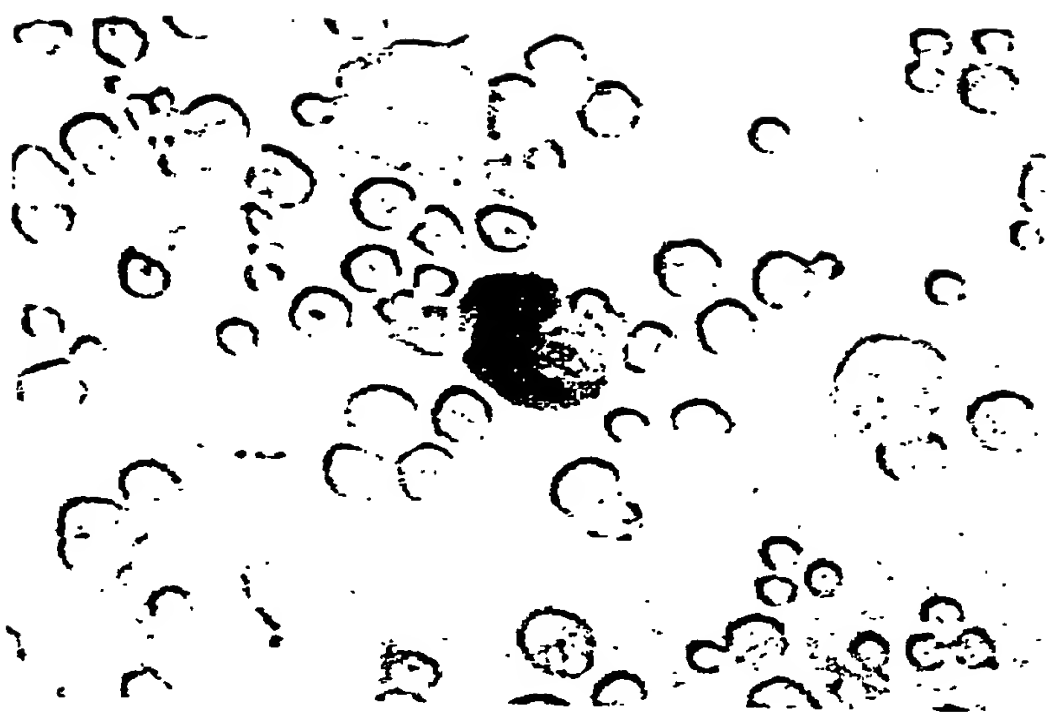


Fig. 7

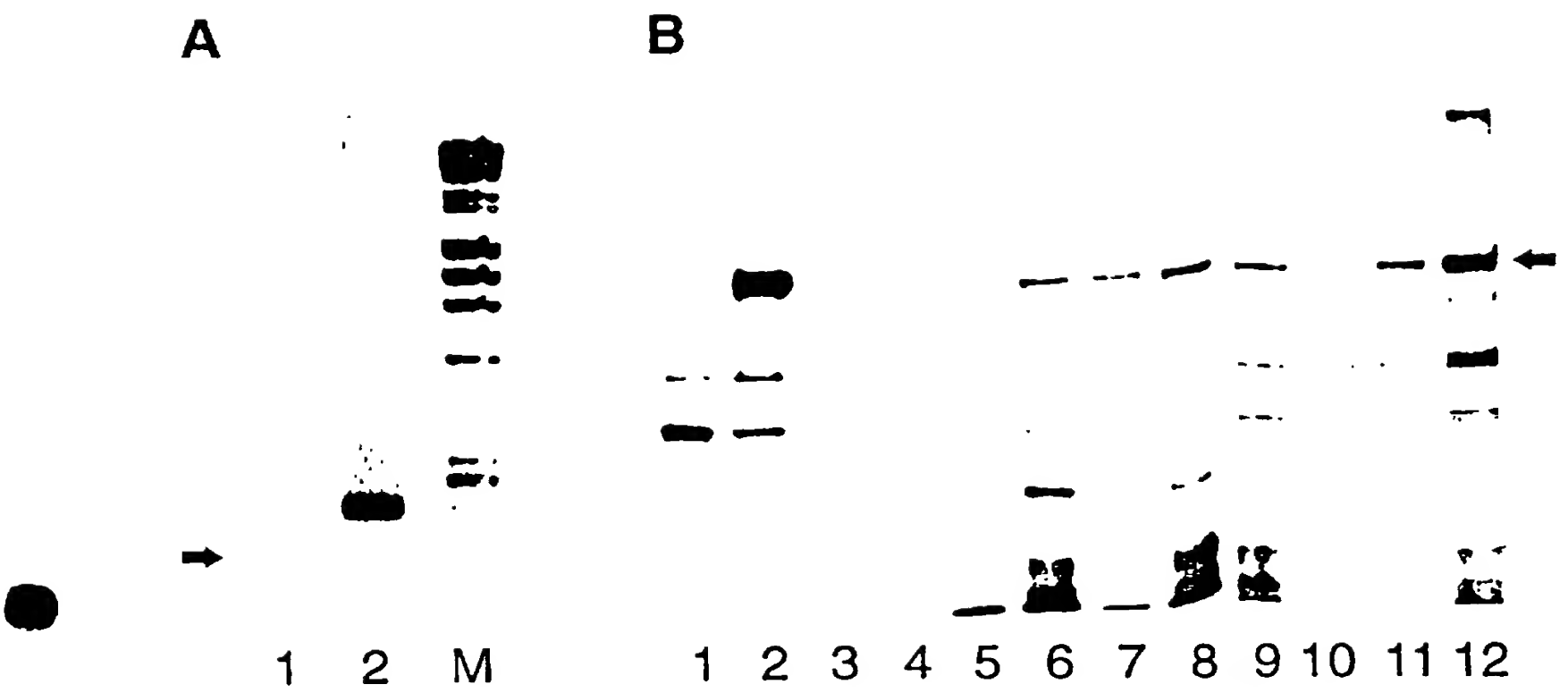
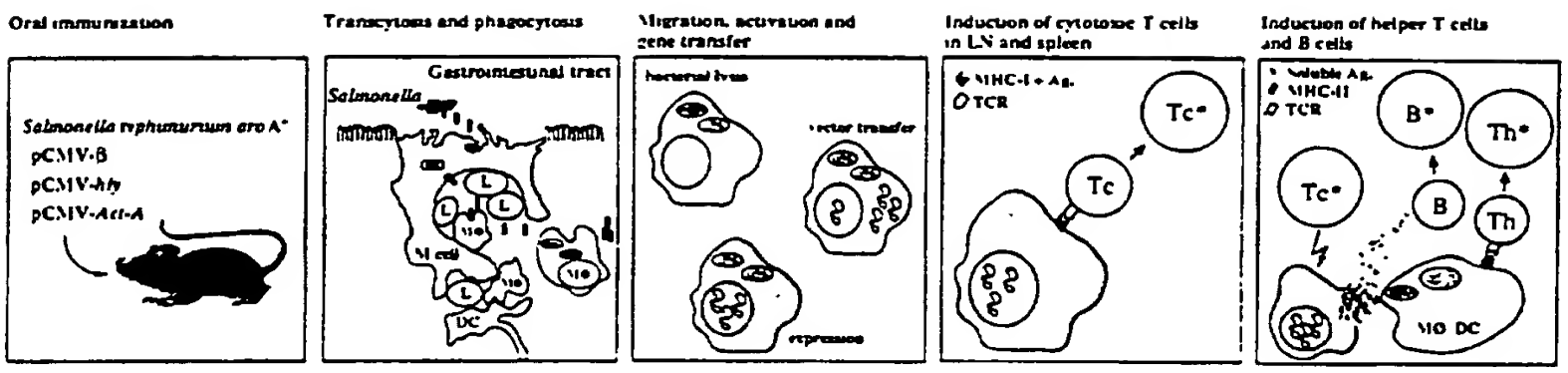


Fig. 8


Oral immunization
Salmonella typhimurium aro A⁻
pCMV-β
pCMV-hly
pCMV-Act-A
Transcytosis and phagocytosis
Gastrointestinal tract
Salmonella
M cell
DC
Migration, activation and gene transfer
bacterial lysis
vector transfer
expression
Induction of cytotoxic T cells in LN and spleen
MHC-I + Ag.
TCR
Tc
Tc*
Induction of helper T cells and B cells
MHC-II
TCR
B
B*
Th
Th*
Tc*
APC

Summary

An attenuated strain of *Salmonella typhimurium* has been tested as vehicle for oral genetic immunization. Eukaryotic expression vectors containing the genes for β-galactosidase, or truncated forms of ActA and listeriolysin - two virulence factors of *Listeria monocytogenes* - that were controlled by an eukaryotic promoter have been used to transform the strain *S. typhimurium aroA*. Multiple or even single immunizations with these transformants induced a strong cytotoxic and helper T cell response as well as an excellent antibody response. Multiple immunizations with listeriolysin transformants protected the mice completely against a lethal challenge of *L. monocytogenes*. Partial protection was already observed with a single dose. ActA appeared not to be a protective antigen.

The strength and the kinetics of the response suggested that the heterologous antigen were expressed within the eukaryotic host cells following transfer of plasmid DNA from the bacterial carrier strain. Transfer of plasmid DNA could be unequivocally shown *in vitro* using primary peritoneal macrophages. The demonstration of RNA splice products and expression of β-galactosidase in the presence of tetracycline - an inhibitor of bacterial protein synthesis - indicated that the gene was expressed by host cells rather than bacteria. Oral genetic immunization with *Salmonella* carriers provides a highly versatile system for antigen delivery, represents a potent system to identify candidate protective antigens for vaccination, and permits efficacious generation of antibodies against virtually any DNA segment encoding an open reading frame.